Differential expression of gap-junction gene connexin 31 in seminiferous epithelium of rat testes

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Received 22 March 1999; received in revised form 12 May 1999

Abstract **Spermatogenesis, a tightly regulated developmental process of male germ cells in testis, is associated with temporal and spatial expression of certain gap-junction connexins. Our findings by RT-PCR indicate that the Cx31 gene is expressed in testis tissue of adult and postnatal rats. During the postnatal spermatogenic process, the Cx31-specific signal became detectable at 15 dpp and onward by in situ hybridization, and apparently localized in the basal compartment of seminiferous epithelium where active spermatogonia and early primary spermatocytes reside. No signal was found in the luminal region. In adult testes, spermatids of elongation phase were also Cx31 positive. Immunohistochemical analysis with mouse anti-Cx31 antibody gave a similar staining pattern, providing further evidence that the gap-junction protein is abundant in the basal seminiferous epithelium, in accordance with the cellular distribution of Cx31 mRNA. These results represent the first demonstration of Cx31 expression at both transcriptional and protein levels in the seminiferous epithelium of rat testes. Thus, Cx31 may play a role in cell-cell communication during spermatogenesis.**

Key words: Connexin 31; Gap junction; Germ cell; Seminiferous epithelium; Spermatogenesis

1. Introduction

Connexins are gap-junction membrane proteins; six connexin subunits assemble into a hemi-channel named connexon, and two connexons align to make a complete intercellular channel [1]. Functionally, these gap-junction channels allow transport of solutes between adjacent cells, intercellular communication, and cellular synchronization, which play an important role in maintaining homeostasis within tissues. Different connexins are expressed in different tissues. Some are cell type specific while others are tissue specific. Connexin 31 (Cx31) is a member of the connexin gene superfamily that has recently been identified and cloned in rat [2], mouse [3], chicken [4], and human [5]. Members of the Cx31 family consist of about 270 amino acids with a predicted molecular weight of ~31 kDa and are closely related (82–90% identity) to one another, except for the avian Cx31 which is similar to the mammalian Cx26 [4]. Expression of the Cx31 gene has been described mainly in extraembryonic tissues, placenta, skin, kidney, Harderian gland, and eye [2–8]. At present, there are at least four Cx31 mRNA transcripts of 1.7, 1.9, 3.4 and 2.3 kb in sizes identified in rodents, and three transcripts, 1.8, 1.9, and 2.2 kb, have been found in human.

Spermatogenesis is a complicated series of events that transform spermatogonia into spermatozoa after mitotic and meiotic cell divisions. The Sertoli cells provide mechanical support, protection, and nutrients for development of the germ cells during the entire process. They are connected to each other and also to the germ cells through various specialized cellular gap junctions. It has been demonstrated that several connexins are distributed within the testis in a specific pattern. So far, five connexin members, Cx26, Cx32, Cx33, Cx37 and Cx43 have been described in the rat testes [9–11]. The Cx31 mRNA transcripts were first found in the mouse testis by Northern blotting [3]. However, no further evidence has been available to support this observation, and the location of Cx31 expression in murine testis remains to be defined. The present study investigated the expression and cellular localization of Cx31 by RT-PCR, in situ hybridization, and immunohistochemical analysis during spermatogenesis in postnatal and mature rat testes.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats obtained from the Laboratory Animal Unit, University of Hong Kong, Hong Kong, were fed with normal rat chow and water ad libitum. Testis, liver, and kidney tissues were collected from adult and postnatal rats at ages of 5, 10, 15 and 18 dpp, after killing by cervical dislocation. The ethical guidelines set forth by the University's Committee on Using Live Animals for Teaching and Research were followed strictly. Resected tissues from testes, liver, and kidney were either immediately snap frozen in liquid nitrogen for RNA extraction, or fixed in Bouin's solution for immunohistochemical analysis and in situ hybridization, respectively.

2.2. Cx primers, mRNA, and RT-PCR

Primers specific to the rat connexin (Cx31, Cx33, and Cx43) genes were designed using the Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA). The β-actin gene was an internal control. Sequences and properties of the primers were listed in Table 1. In general, the primer sequences were derived from the C-terminal coding region that was found to be unique to each connexin member. Primer specificity was further examined by sequence comparison against the mouse and human Cx homologs as well as other related Cx genes by the BLASTA (http://www.ncbi.nlm.nih.gov/BLAST). Amplification of cDNA by RT-PCR was achieved by employing poly $(A)^+$ mRNA as templates, and RT-negative control (the same PCR procedures but without reverse transcription) was included in each RT-PCR reaction. Oligonucleotides were synthesized by Life Technologies, Inc. (Gaithersburg, MD, USA).

Poly $(A)^+$ mRNA was isolated from frozen tissues using guanidinium thiocyanate and oligo (dT)-cellulose chromatography (QuickPrep Micro mRNA Purification Kit; Pharmacia, Uppsala, Sweden). The first-strand cDNA synthesis was carried out in 20 μl of reaction

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Abbreviations: Cx, connexin; dpp, days postpartum; ISH, in situ hybridization; RT-PCR, reverse transcription-polymerase chain reaction

0014-5793/99/$20.00
PII: S0014-5793(99)00714-0

Table 1
Properties of PCR primer sets

Primer	Location	T^m (°C)	Sequence	Expected size of amplicon
Cx31-forward	320–337	56.2	5′-AGAAGCACGGGGAGCAT-3′	442 bp
Cx31-reverse	762–741	55.4	5′-TACTGCTTCCAGATCACCACTC-3′	
Cx33-forward	669–698	65.9	5′-CTTGAATGTCATCGAGCTGTTCTATGTCTT-3′	169 bp
Cx33-reverse	837–808	66.5	5′-CACCGGGACTACCTGATCACTAGAACATAT-3′	
Cx43-forward	739–759	69.4	5′-TACCACGCCACCACTGGCCCA-3′	294 bp
Cx43-reverse	1032–1009	71.5	5′-GGCATTCTGGTTGTCGTCGGGGAA-3′	
β-actin-forward	1035–1058	64.2	5′-ACTGTGCCCATCTACGAGGGCTAT-3′	158 bp
β-actin-reverse	1192–1169	64.2	5′-TCTTTGATGTCACGCACGATTTCC-3′	

mixture containing 50–100 ng of extracted mRNA, 0.2 μg of random hexamers, 200 mM DTT and 11 μl of Bulk First-Strand cDNA Mix (dNTPs, porcine RNAguard, RNase/DNase-Free BSA, and MULV reverse transcriptase) (Pharmacia). Samples were incubated at 37°C for 60 min and reactions were stopped at 95°C for 5 min. The resulting cDNA preparations were stored in aliquots at −80°C. RT-negative control was without the addition of MULV reverse transcriptase.

For PCR, 1–2 μl of the reverse transcribed cDNA template (approximately 0.1–0.2 ng) was added to a final volume of 50 μl reaction buffer containing 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM dNTPs, 0.8 μM each primer, and 2 U *Taq* DNA polymerase (Boehringer Mannheim, Indianapolis, IN, USA) in 10 mM Tris-HCl (pH 8.3). After an initial denaturation at 94°C for 5 min, the reaction was subjected to 30 cycles of 94°C for 30 s; 55–66°C (primer T^m listed in Table 1) for 30 s; and 72°C for 45 s. A buffer control that contained no DNA template was included in each PCR assay as surveillance for interior contamination. The PCR products were analyzed by electrophoresis in 2% agarose gel, stained with ethidium bromide, and visualized by UV illumination.

2.3. In situ hybridization (ISH)

A 33-mer Cx31-specific antisense probe corresponding to the C-terminal region (sequence 609–641 bp: 5′-AAT CCT GTG GAA GAT GAG GTA GCA GAT CTC ACA-3′) was synthesized as previously published [8]. The probe was labeled with digoxigenin at the 3′-end (DIG Oligonucleotide Tailing Kit, Boehringer Mannheim). Briefly, 100 pmole (or 1 μg) of oligonucleotide was added to 20 μl reaction mixture containing 250 mM potassium cacodylate, 0.25 mg/ml BSA, 25 mM $CoCl_2$ solution, 1 mM DIG-dUTP, 10 mM dATP, and 50 U terminal transferase in 25 mM Tris-HCl (pH 6.6). After incubation at 37°C for 15 min, the reaction was stopped by adding 2 μl of glycogen (2 μg/ml) and EDTA solution (0.2 M; pH 8.0).

Bouin-fixed, paraffin-embedded tissue sections (7 μm thickness) were mounted onto 3-aminopropyl-triethoxysilane coated slides, dried at 37°C overnight, deparaffinized in fresh xylene and graded alcohol series. The standard sections included section of postnatal testes (5, 10, 15 and 18 dpp), mature testis, kidney and liver. Sections were brought to room temperature and then pretreated with 7 μg/ml proteinase K, post-fixed with 4% paraformaldehyde and acetylated with 0.1 M triethanolamine-HCl buffer (pH 8.0) containing 0.25% (v/v) acetic anhydride.

For hybridization, the sections were added with 100 μl of the DIG-labeled antisense Cx31 probe diluted (1:1000) in the prehybridization buffer which contained 50% (v/v) formamide, 10% dextran sulfate, 50 mM DTT, 50 mM phosphate buffer, 2×SSC, 1×Denhardt's solution, 5 μg/ml polydeoxyadenylic acid, 100 μg/ml polyadenylic acid, 250 μg/ml yeast RNA, and 500 μg/ml denatured/sheared herring sperm DNA. After coverslipping and overnight incubation at 37°C in a humidified chamber, the slides were subjected to stringent washes with (i) 2×SSC for 15 min at 37°C twice, (ii) 1×SSC for 15 min at 37°C twice, and (iii) 0.25×SSC for 15 min at 37°C twice. After blocking with normal goat serum, the hybridized sections were incubated with sheep anti-DIG antibody conjugated with alkaline phosphatase (1:500 dilution; Boehringer Mannheim) at 37°C for 2 h. Substrate 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium solution containing 1 mM levamisole (Sigma Co., St. Louis, MO, USA) was used for color development. The section adjacent to the section used for in situ hybridization was stained with hematoxylin and eosin for histology observation to provide mirror image of the hybridized section. Images were viewed with Nikon epifluorescent upright microscope E600 (Nikon, Tokyo, Japan), captured with 3-CCD color camera DC-330 (DAGE-MTI, Inc., Michigan City, IN, USA), and analyzed by the Metamorph v3.0 (Universal Imaging Corporation, West Chester, PA, USA).

2.4. Cx31 antibody and immunohistochemistry

Specific antibodies directed against the rat Cx31 were raised in female BALB/c mice according to previous procedures [12] by immunization with synthetic peptides $(His)_6$PADDKLQASAPSLTPI couple to agarose beads. Peptide sequence was derived from the specific COOH-terminal domain (a.a. sequence: 255–270) of rat Cx31 molecule. Prior to each injection, 200 μg of the peptide was allowed to bind at room temperature for 30 min to equal volume of nickel-bound nitrilotriacetic acid (Ni-NTA) agarose (QIAGEN, Chatsworth, CA, USA) that was prewashed with sterile PBS. The peptide conjugate was emulsified with an equal volume of complete or incomplete Freund's adjuvant (Sigma) for dual subcutaneous and intraperitoneal administrations. The antibody specificity was characterized by Western blot against the testis lysates and by immunofluorescence binding assay to the solid-phase Cx31 peptide conjugate according to published procedures [13].

Paraffin sections (4 μm) of adult rat testis mounted on objective slides were treated with 14 μg/ml of proteinase K at 37°C for 25 min. After washing with PBS and blocking with 3% BSA in PBS, the sections were allowed to react at 4°C overnight, with either normal preimmune mouse serum or anti-Cx31 antibodies. After gentle washings, the sections were incubated with 1:500 dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG (H+L) antiserum (Zymed Lab, S. San Francisco, CA, USA) at 37°C for 1 h. Bound antibody was revealed by addition of diaminobenzidine substrate (DAB; Sigma) in hydrogen peroxide solution for color development, and the sections were counterstained with hematoxylin and eosin.

3. Results

3.1. RT-PCR analysis of Cx31, 33, and 43 expression in rat testis

RT-PCR was performed to study the expression of different connexin genes in rat testis during spermatogenesis. Fig. 1A showed the RT-PCR results and the expected PCR amplicons were detected (442 bp for Cx31, 169 bp for Cx33, 294 bp for Cx43, and 158 bp for β-actin). Similar to other reports, Cx33 and Cx43 were strongly expressed in adult rat testes. In addition, Cx31 also gave a positive signal in the RT-PCR, albeit with a relatively faint band. Similar results were obtained after repeating twice. No signal was detected in the RT-negative panel in which the reverse transcription step was omitted. This excluded the possibility of falsification by amplification of genomic DNA.

To further investigate the expression of Cx31 during spermatogenesis, rat testes at different postnatal stages (5, 10, 15 and 18 dpp) and adult age, as well as other tissues such as kidney and liver were analyzed by RT-PCR. As shown in Fig. 1B, the expected Cx31 amplicons appeared in the lanes of testes and kidney, but not of the liver which was a negative control. The identity of the Cx31 amplicon was verified by

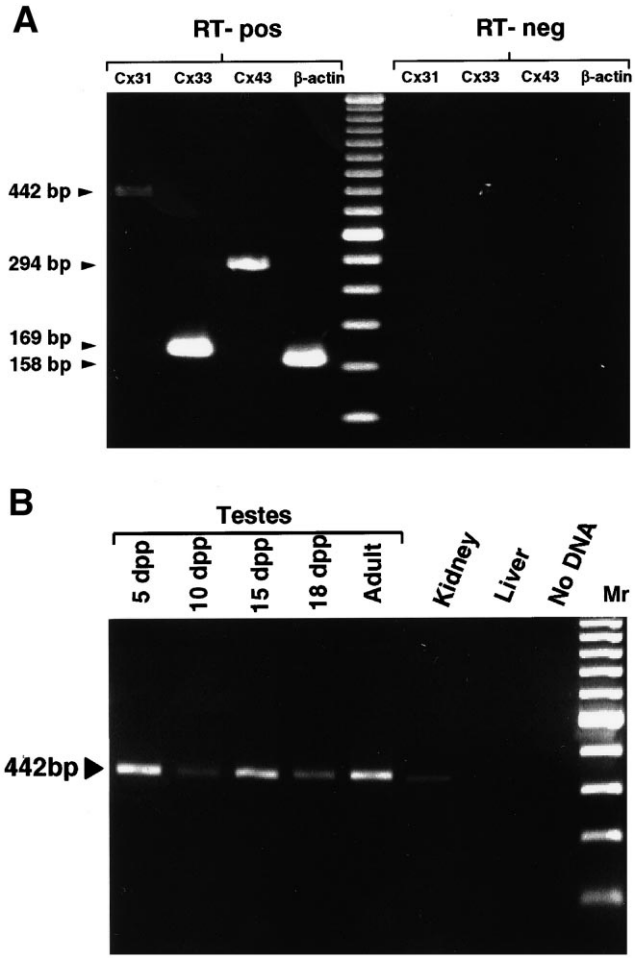

Fig. 1. A: Expression of connexins in rat testis. Poly A^+ mRNA isolated from adult testes were subjected to PCR amplification using primers complementary to the C-terminal coding sequences, with or without prior reverse transcription. The predicted amplicon sizes were 442 bp for Cx31, 169 bp for Cx33, 294 bp for Cx43, and 158 bp for β-actin, respectively. The marker was a 50-bp DNA ladder in the center lane. B: RT-PCR analysis of Cx31 mRNA expression in rat testis. Arrows indicated the expected size of the PCR products (442 bp). The pattern suggested that Cx31 mRNA was present from postnatal age: 5, 10, 15 and 18 dpp up to mature stages. Control tissues are kidney (positive) and liver (negative). Mr, a 100-bp DNA ladder.

restriction digestion with *Kpn*1 at a unique restriction site within the amplicon, generating the expected bands of 186 and 258 bp (data not shown).

3.2. *Expression of Cx31 gene in rat testes during spermatogenesis*

By in situ hybridization with Cx31-specific probe, positive hybridization signals were observed in testes from adult rats (Fig. 2b). Essentially, the Cx31 signal was localized to two major cell types – the active spermatogonia and the early primary spermatocytes (leptotene and zygotene) in the basal compartment of seminiferous epithelium where spermatogenesis was initiated. The adluminal spermatids at the elongation phase in adult testis were also found positive, but not in earlier stages. Sertoli cells did not show a detectable hybridization signal for Cx31 in the same tissue section. Liver as a negative control tissue for Cx31 was negative in the ISH assay (Fig. 2d).

Postnatal rat testes at different stages (5 to 20 dpp) were examined similarly by ISH for the expression of Cx31 gene during spermatogenesis. As shown in Fig. 3, the hybridization signals were more prominent with increase in postnatal age when more spermatogonia underwent spermatogenesis (i.e. increasing number of germ cells). However, the Cx31 ISH signal was hardly detectable at 5 days when Sertoli cells were predominant and actively replicating. Weak hybridization appeared at 10 dpp, and positive signal became more abundant in testes from 15 dpp. Positive ISH signal was also observed at 18 and 20 days (data not shown).

3.3. *Immunohistochemical analysis of Cx31 localization in seminiferous epithelium*

Anti-Cx31 antibody was obtained by immunizing mice with synthetic peptide-agarose conjugate. The Cx31 antibody gave a 31-kDa band in the Western blot, and by immunofluorescence binding assay, stained against the Cx31 peptide-agarose beads (data not shown). Thus, we analyzed the immunohistochemical localization of Cx31 protein in adult rat testis, in order to test whether or not its localization would coincide with the mRNA pattern. Fig. 4 shows that staining of this gap-junction membrane protein was abundant in spermatogonia and early spermatocytes located in the basal compartment of the seminiferous epithelium. High power magnification (Fig. 4c) showed that the immunoreactive Cx31 was localized to cytoplasmic membrane of the male germ cells. This observation correlated with the presence of Cx31 mRNA in this region as shown by ISH. No reaction was observed with the Sertoli or other somatic cells, and not in the control section.

4. Discussion

The present study investigated the expression of gap-junction connexin gene Cx31 in rat testes from postnatal to adult stages during spermatogenesis. First, we have found by RT-PCR that Cx31 is expressed in adult rat testis, together with the other known gap-junction proteins Cx33 and Cx43 which have been reported to be present and served as a control here. Second, the Cx31 gene was also detected in postnatal testes from 5 to 20 dpp. Contrary to the RT-PCR, the Cx31 signal was prominent from 15 dpp of the postnatal development by in situ hybridization, albeit with a weak hybridization result at 10 days. This difference from the RT-PCR result is probably due to a lower level of Cx31 expression in the testes at the early ages when the Sertoli cells outnumber the germ cells. As a result, the Cx31 level was insufficient for a detectable signal by in situ hybridization. Third, both the Cx31 mRNA and protein signals were first observed in the basal compartment of seminiferous epithelium, and the expression was essentially concentrated on particular populations of germ cells, i.e. the active spermatogonia and early primary spermatocytes. The Cx31 gene expression in these particular germ cells was also observed throughout the spermatogenic cycle in adult testes. No signal was found in other germ cells of the later stages except that of spermatids at the elongation phase.

Our finding is the first report on the expression of Cx31 in rat testis; also this is the first demonstration of cellular localization of connexin expressed in male mammalian germ cells. In rat, the prepubertal maturation of seminiferous epithelium starts from approximately 5 dpp [14], when pre-spermatogonia begin to divide and the Sertoli cells are still extensively

replicating [15]. In 10 dpp, spermatogonia enter meiosis. During this process, functional gap junctions are formed between adjacent Sertoli cells, allowing intercellular transport of small biological molecule [16]. From 15 dpp, the Sertoli–Sertoli tight junctions appeared, and a blood testis barrier is established by 20 dpp [17].

In a previous observation, Cx31 was also found in mouse testis [3], but its cellular distribution had not been defined. Here, we demonstrated by in situ hybridization and immunohistochemistry that the Cx31 was expressed in the active spermatogonia and early primary spermatocytes located at the basal compartment of seminiferous epithelium during spermatogenesis. In adult testes, the adluminal elongated spermatids were also found Cx31 positive. The specific spatial and temporal expression of Cx31 in the germ cells of rat testes suggests that the Cx31 function is tightly regulated, and is required in restricted windows both at early and later stages during the spermatogenic cycle. Several human diseases have been related to connexin mutations, such as the X chromosome-linked form of Charcot-Marie-Tooth disease [18], the

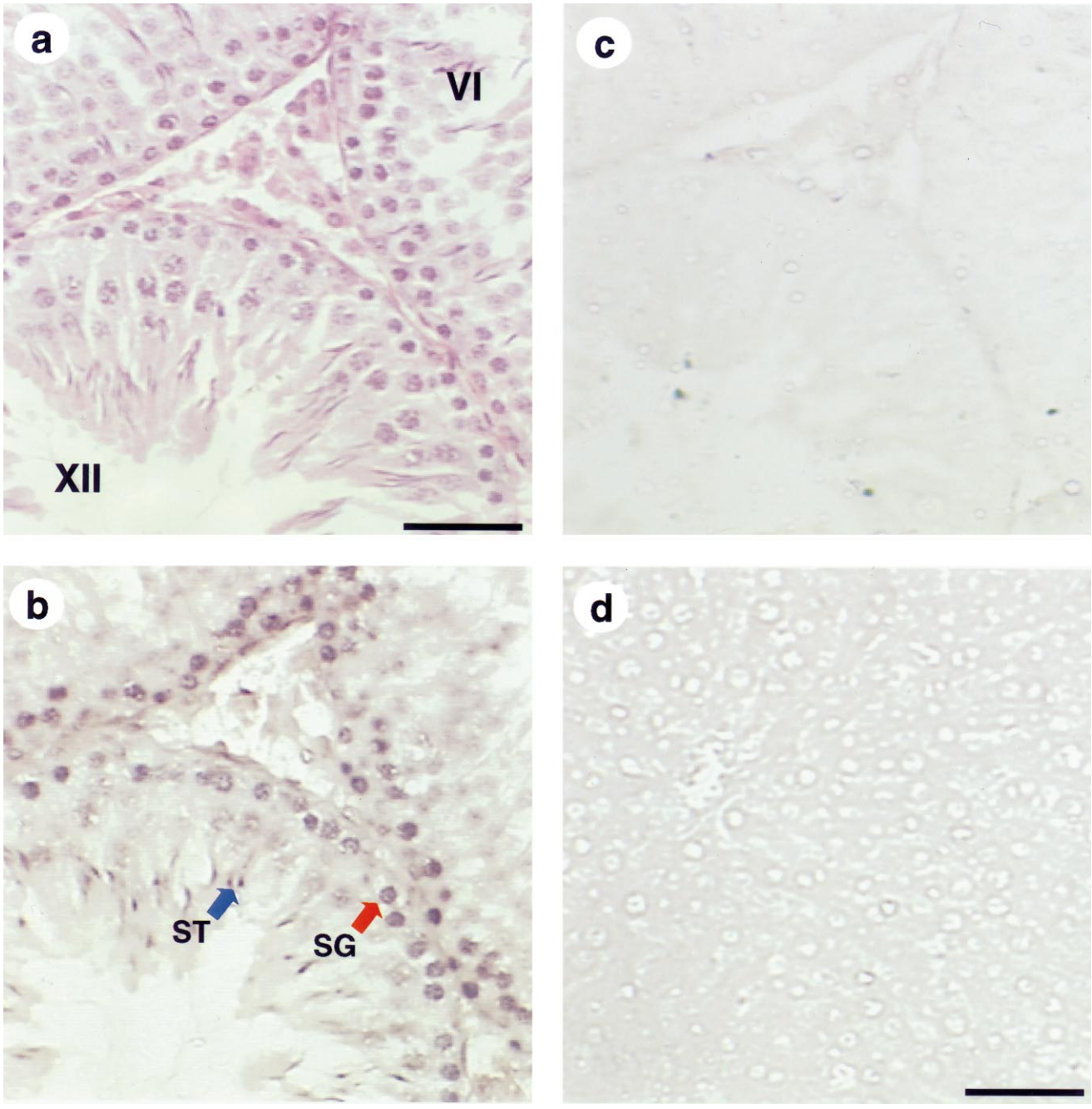

Fig. 2. Cellular expression of Cx31 gene in seminiferous epithelium. Successive sections (mirror images) of adult testis. a: Hematoxylin and eosin staining to show the histology (stages VI and XII seminiferous tubules) of the section adjacent to the hybridized section; b: hybridized with the antisense Cx31 oligonucleotide probe; and c: hybridized with control probe solution. d: Hybridization with the antisense Cx31 probe in liver tissue section. Early spermatocytes (leptotene or zygotene) and spermatogonia (SG) in the basal region and the adluminal elongated spermatids (ST) are shown by arrows. (Magnification, ×200; bar, 5 μm.)

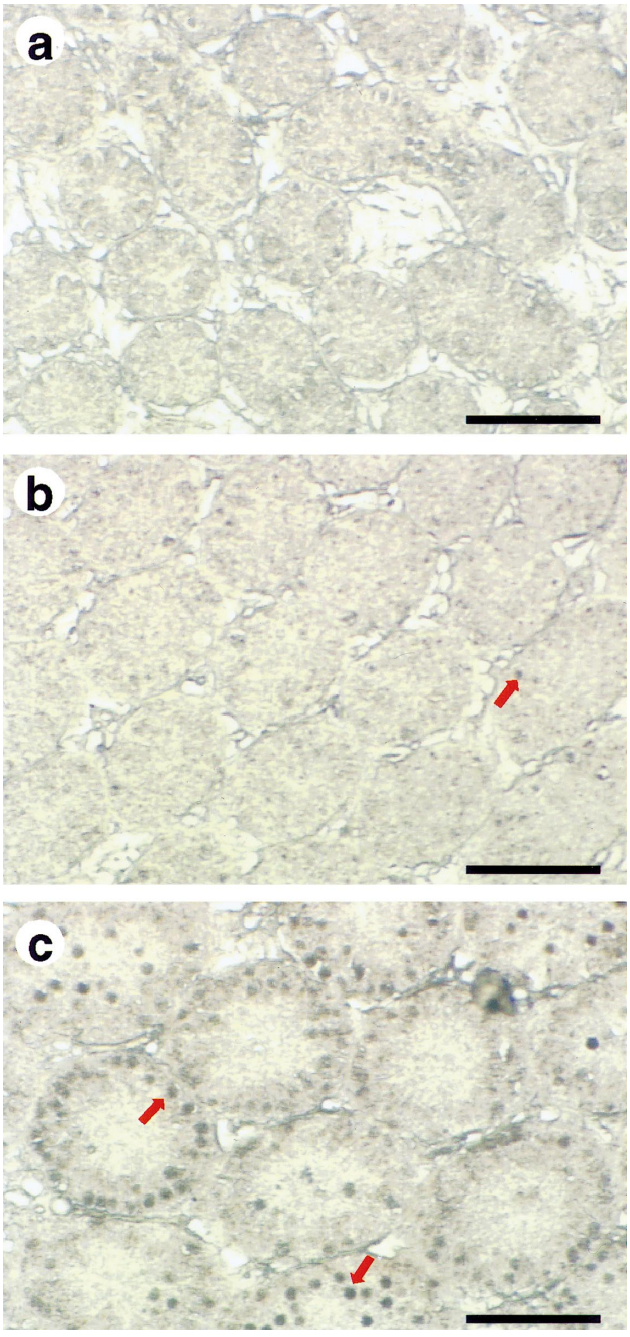

Fig. 3. In situ hybridization of Cx31 gene in rat testis at distinct postnatal stages. a: 5 dpp, b: 10 dpp, and c: 15 dpp of postnatal rat testes. Positive signal was indicated by arrows. (Magnification ×200; bar, 10 μm.)

common non-syndromic neurosensory autosomal recessive deafness [19], and developmental anomalies of the cardiovascular system [20]. Thus, it is of great clinical interest to determine the molecular mechanisms of male subfertility in humans, and whether or not the Cx31 and other testicular connexins have mutations and unusual expression that would lead to spermatogenic dysfunction. Further investigations to elucidate the physiologic role(s) of Cx31 in spermatogenesis are under way.

Rat testes express other connexin members, Cx26, Cx32, Cx33, Cx37 and Cx43 [9–11,21,22]. Among them, Cx33 is

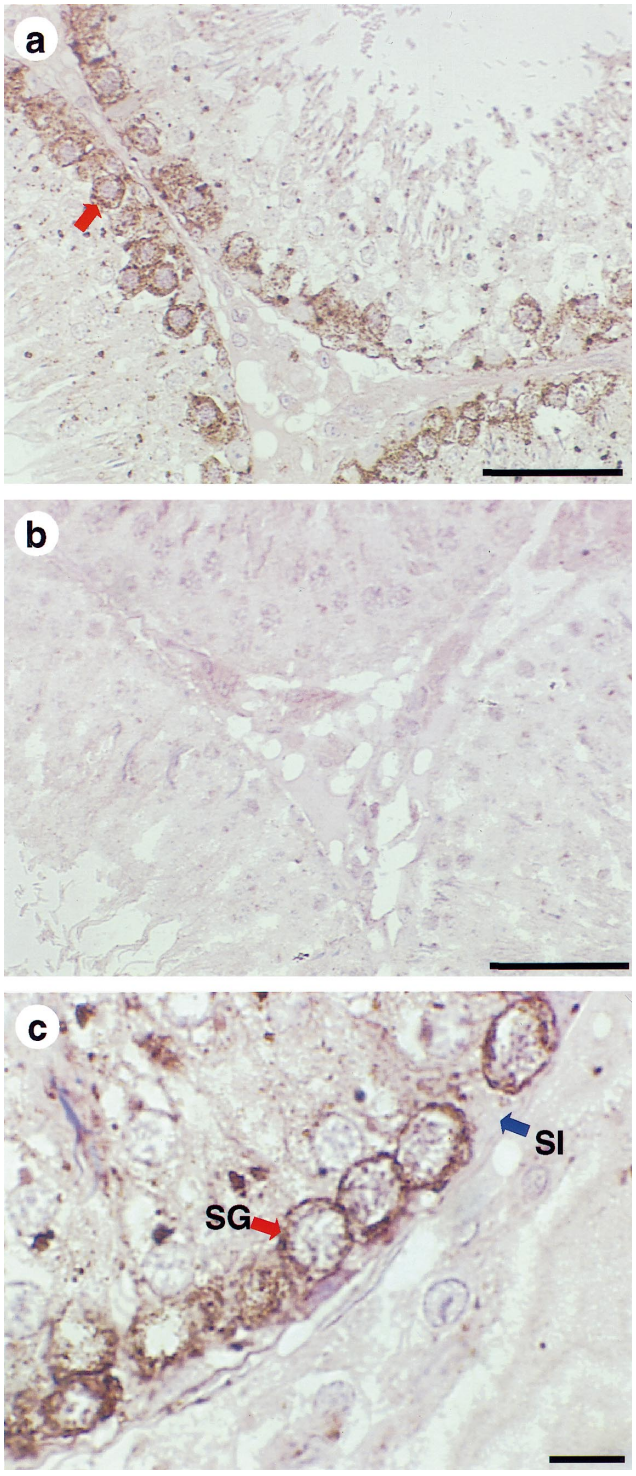

Fig. 4. Immunohistochemical analysis of Cx31 localization in rat testis. Paraffin sections (4 μm thick) of adult rat testes stained with: a: mouse Cx31-specific antibodies, and b: normal mouse antibodies. (Magnification, ×400, bar, 10 μm.) c: High power magnification (×1000) of Cx31-immunostained section with special focus on the cytoplasmic membrane of spermatogonia (bar, 1 μm). Positive spermatogonia (SG) and negative Sertoli cells (SI) were shown by arrows. Peroxidase-conjugated rabbit anti-mouse IgG (H+L) and DAB substrate were used for color development to visualize bound antibody complex. Sections were counterstained with hematoxylin and eosin.

known to be testis specific, and is found on Sertoli cell surface starting from 15 dpp and accumulating gradually in all seminiferous tubules through 28 dpp in the postnatal stages. In adult testes, the Cx33 expression is weak in stage IX–XIV tubules where the elongated spermatids are dominant. Likewise, expression of Cx43 in rat testes is also spermatogenic stage-dependent and it is the only type of connexin present in the Leydig cell gap junctions [9]. Cx43 is also present in the Sertoli–Sertoli occluding junctions of stage I–VIII tubules, but not in the stage IX–XIV tubules. Unlike the Cx43, expressions of Cx26 and Cx32 are localized to the apical regions of the seminiferous epithelium in mature testis. Cx37 is restricted to the endothelial cells of blood vessel [10]. Our present study indicates that contrary to other testicular connexins, Cx31 is expressed in germ cells of certain stages during spermatogenesis: spermatogonia, early spermatocytes, and elongated spermatids. It is indicative that Cx31 may serve particular function(s) different from other connexins.

Connexin molecules can form homotypic and/or heterotypic gap junctions. A model based on electron microscopy and X-ray crystallography has revealed that the gap-junction channel is formed by the end-to-end interaction of two connexons [23,24]. Docking between connexons involves interactions between the extracellular loops, in which the six conserved cysteines influence the selectivity of docking between different connexons. By means of Lucifer yellow transfer analysis, it was shown that the Cx31-transfected Hela cells were unable to communicate with other connexin members (Cx26, Cx32, Cx37, Cx40, Cx43, and Cx45) [25]. Thus, Cx31 forms homotypic gap junction only. One possible reason is that the spacing arrangement of cysteine residues in the E2 loop of Cx31 is distinct from the other connexins, sterically hindering the precise alignment of Cx31 with other connexons to form a functional channel. In accordance, the restricted expression pattern of Cx31 at the seminiferous epithelium in certain developmental stages may play a unique role in the germ cell-germ cell communication in the process of spermatogenesis.

Acknowledgements: This study was performed by B.W.M. for partial fulfillment of her M. Phil. degree. B.W.M. is a recipient of postgraduate studentship from the University Grants Committee, H.K. Grant supports (to J.M.L. and W.S.B.Y.) were from the CRCG of the University of Hong Kong. We gratefully acknowledge the critical reading of this manuscript by Drs. Vincent Lui and K.M. Yao of the Department of Biochemistry.

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